

Reference assays for *Clostridium difficile* infection: one or two gold standards?

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ABSTRACT

Accurate diagnosis of *Clostridium difficile* infection (CDI) is essential for optimal treatment, prevention and control. There are two reference assays for CDI diagnosis: the cell cytotoxicity assay (CCTA) and toxigenic culture (TC). Importantly, these tests actually detect different targets: CCTA detects the presence of *C difficile* toxins (primarily toxin B, but also toxin A), whereas TC detects the presence in the stool of *C difficile* with the potential to produce toxin. Not surprisingly studies comparing the results of these assays show imperfect agreement. Thus, a faecal sample may be CCTA negative but TC positive, and this raises the crucial question about the clinical significance of the presence of *C difficile* with the capacity to produce toxin but no actual detectable free toxin. A positive TC result indicates that a patient with diarrhoea is potentially infectious. TC also has the advantage that the cultured isolate is available for typing and for susceptibility testing. In general, however, CCTA has been shown to be a better test for the laboratory confirmation of CDI, although additional culture may be needed to optimise sensitivity. Crucially, when these reference assays are used to determine the accuracy of alternative diagnostic tests, care should be taken to compare methods with their appropriate standard (ie, compare tests that target equivalent end-points). Such issues have contributed to the variable and often suboptimal performance of rapid diagnostic tests for CDI. Further research is urgently needed to improve knowledge of the utility of routine diagnostic tests in CDI and the factors that influence their performance.

INTRODUCTION

Clostridium difficile infection (CDI) is a potentially life threatening, usually healthcare-associated infection that causes considerable morbidity and mortality. CDI has become more common in many countries during the last decade, and may be increasing in severity associated with the introduction of epidemic strains. Most infections are acquired in hospitals, where CDI frequently afflicts vulnerable patients already or recently receiving antibiotics. In the UK, national reporting of CDI is mandatory, and targets have been set to reduce the incidence of cases. Such enhanced surveillance has underpinned the decrease in reported cases seen in England between 2008 and 2009.¹

Accurate diagnosis of CDI is vital for patient management and infection control. Correct diagnosis is also essential for the attainment of reliable surveillance data, and specifically to enable efficient tracking of infections, comparisons between institutions as part of performance management, and accurate determination of the efficacy of

interventions to reduce CDI risk. False-negative test results may lead to patients not being treated appropriately for CDI, and these may not be appropriately isolated to reduce the risk of transmission. However, false-positive results can similarly have important consequences, including inappropriate cessation of antibiotics, unnecessary initiation of CDI treatment, and failure to investigate alternative diagnoses. In some instances patients who have false-positive results may be cohorted with true CDI cases (eg, on *C difficile* wards), so potentially exposing these individuals to increased risk of acquiring a genuine infection. Furthermore, the assessment of the accuracy of tests to confirm the diagnosis of CDI depends critically on the choice and performance of a reference standard assay. Without the appropriate choice and performance of a reference assay to define true positives and true negatives, it is not possible to accurately develop and assess new tests. In turn, use of suboptimal tests cloud our understanding of the diagnosis, epidemiology and control of CDI, not least because we cannot reliably distinguish on clinical grounds between *C difficile* and other infective or non-infective causes of diarrhoea.

REFERENCE ASSAYS FOR *C DIFFICILE*

The cell cytotoxicity assay (CCTA) has been traditionally regarded as the reference ('gold standard') assay for the laboratory confirmation of CDI. This assay relies on the detection of a cytopathic effect in cell culture that is neutralised by the presence of antibodies to *C difficile* toxins. Cells (eg, Vero or Hep2 cells) are cultured in the presence of a faecal filtrate, with and without the presence of neutralising antitoxin antibodies. These cultures are examined microscopically at 24 and 48 h for evidence of a cytopathic effect (cell rounding due to apoptosis) that is prevented by the specific antitoxin. CCTA requires the ability to perform cell culture, which is being used much less frequently in laboratories as nucleic acid amplification tests are used more widely, and a degree of expertise to recognise a cytopathic effect. There are commercially available frozen cells, such as human foreskin fibroblasts (Diagnostic Hybrids, Athens, OH, USA). These cells are more convenient, but their use needs validation. Clearly, current CCTAs are relatively slow, although limited data suggest that positive results may be obtainable after shorter incubation periods²; nevertheless, in practice, results are only available from the day following sample submission.

Some authors consider that culture of *C difficile* followed by a cell cytotoxicity (or possibly other

toxin) assay of suspect colonies (toxigenic culture, TC) is a more sensitive test for CDI.^{3,4} TC relies on the anaerobic culture of *C difficile* from faeces, usually preceded by alcohol shock of the faecal sample to remove vegetative bacteria (that may overgrow *C difficile*), so selecting the hardy spores of *C difficile*. The faecal sample is then cultured on specific agar plates (eg, *C difficile* cefoxitin cycloserine egg-yolk, CCEY) for at least 48 h, and typical colonies are identified. There are isolates of *C difficile* that do not produce toxins, and so it is necessary to confirm that suspect isolates are actually toxigenic. Cytotoxigenic culture may therefore take 4–5 days to confirm the presence of a toxigenic isolate, making this somewhat impractical for routine diagnostic use. A variant approach involves testing colonies directly for evidence of toxin production using an immunoassay, which (although not validated for such use) can produce a positive TC result 1–2 days after the sample is received.^{3,5} Also, similar to criticisms of CCTA, TC requires technical expertise to culture and identify *C difficile* reliably. There are advantages associated with culture,³ including the ability to type isolates and to perform antibiotic susceptibility testing.

A number of studies have compared the performance of CCTA and TC (table 1). It is clear from these that, in general, TC detects more positive samples than the CCTA; CCTA has a sensitivity of about 75–85%^{6–13} compared with TC. These studies also showed that a variable proportion of cases (2–15%)^{6–13} are TC negative, but positive by CCTA. Such observations may be due to the lack of sensitivity of culture methods, and this is exacerbated by the variable performance of *C difficile* selective media.¹⁴ Notably, the levels of agreement between CCTA and TC vary greatly between test sites. It is therefore probable that there are key factors that affect the performances of the reference assays. For example, sample freshness, patient pretreatment with metronidazole or vancomycin, the timing of the assay in relation to symptom onset, or testing all faecal samples as opposed to only those in patients with clear evidence of diarrhoea (≥ 3 unformed stools in 24 h) may influence test results. As these factors vary greatly between sites and over time, and the proportion of discordant results may change, making it impossible to generalise from the results of individual studies.

The fact that TC is more often positive than CCTA, along with perceived technical difficulties in performing CCTA, has

led some to claim that the former assay is the 'real gold standard',⁴ especially for determining the accuracy of alternative diagnostic tests for CDI. There is some irony to this debate considering that in many diagnostic laboratories neither of the reference assays is used for the routine diagnosis of CDI. Crucially also, available diagnostic tests for CDI actually detect different targets (table 2 and discussed below). CCTA detects the presence of *C difficile* toxins (primarily toxin B, but also toxin A), whereas TC detects *C difficile* bacteria or spores that have the potential to produce toxin. Thus, a faecal sample may be CCTA negative but TC positive, and this raises the crucial question about the clinical significance of the presence of *C difficile* with the capacity to produce toxin but no actual detectable free toxin. As toxin-producing *C difficile* can be cultured from about 2% of the general population and 7–25% of hospitalised patients, a TC-positive result may occur in the absence of CDI (see below). Also, not all *C difficile* strains produce the major toxins (A and B) that mediate disease. Not surprisingly, therefore, studies comparing the results of culture, CCTA and TC tests show imperfect agreement.

The development of CDI is a multistage process and is summarised in figure 1. The patient first needs to be exposed to (usually the spores of) *C difficile*, which usually occurs close to the onset of CDI, but occasionally may reflect longer term carriage. Second, there needs to be overgrowth of *C difficile*, and this normally occurs after disruption of the normal bowel flora by antibiotic therapy. Finally, there needs to be toxin production that causes diarrhoea±colitis. *C difficile* may be cultured from the faeces of up to 30% of inpatients without diarrhoea who have been in hospital for several weeks.^{15,16} It remains unclear whether a patient with *C difficile* culture positive faeces is transiently carrying or actually colonised by the bacterium. Thus, the reference assay may be positive at different stages before and during the development of CDI.

EPIDEMIOLOGY OF *C DIFFICILE* AND REFERENCE ASSAYS

A full understanding of the epidemiology of *C difficile*, particularly with regards to the detection of cytotoxin or culture of cytotoxigenic *C difficile*, is essential to interpret the results of the reference assays. Since the early 1980s, there have been many studies where faeces have been cultured for cytotoxigenic *C difficile* from individuals with and without diarrhoea.

Table 1 Studies comparing of 'gold standard' methods for *C. difficile* showing sensitivity, specificity and kappa values

Reference	Sensitivity	Specificity	κ (SE)	Patient group
Cytotoxigenic culture as reference standard				
Van pouke <i>et al</i> (2001) ¹¹	0.743 (55/74)	0.997 (291/292)	0.814 (0.052)	Stool sample with request for <i>C difficile</i> toxin
Barbut <i>et al</i> (2009) ¹²	0.758 (25/33)	1.000 (267/267)	0.848 (0.058)	Diarrhoeal stool sample with request for <i>C difficile</i> toxin
Kelly <i>et al</i> (1987) ¹⁰	0.684 (65/95)	0.996 (529/531)	0.774 (0.039)	Stool samples received in laboratory
Eastwood <i>et al</i> (2009) ¹³	0.864 (108/125)	0.992 (471/475)	0.890 (0.041)	Stool samples received in laboratory
Fedorko <i>et al</i> (1999) ⁹	0.718 (56/78)	0.981 (565/576)	0.744 (0.039)	Stool sample with request for <i>C difficile</i> toxin
Merz <i>et al</i> (1994) ⁸	0.855 (47/55)	0.994 (639/643)	0.878 (0.038)	Stool sample with request for <i>C difficile</i> toxin
DiPersio <i>et al</i> (1991) ⁷	0.814 (35/43)	0.967 (261/270)	0.773 (0.057)	Stool samples received in laboratory
Schué <i>et al</i> (1994) ⁶	0.790 (49/62)	0.993 (291/293)	0.842 (0.053)	Stool samples received in laboratory
Cell cytotoxicity assay as reference standard				
Van pouke <i>et al</i> (2001) ¹¹	0.982 (55/56)	0.939 (291/310)	0.814 (0.052)	Stool sample with request for <i>C difficile</i> toxin
Barbut <i>et al</i> (2009) ¹²	1.000 (25/25)	0.971 (267/275)	0.848 (0.058)	Diarrhoeal stool sample with request for <i>C difficile</i> toxin
Kelly <i>et al</i> (1987) ¹⁰	0.970 (65/67)	0.946 (529/559)	0.774 (0.039)	Stool samples received in laboratory
Eastwood <i>et al</i> (2009) ¹³	0.964 (108/112)	0.965 (471/488)	0.890 (0.041)	Stool samples received in laboratory
Fedorko <i>et al</i> (1999) ⁹	0.836 (56/67)	0.963 (565/587)	0.744 (0.039)	Stool sample with request for <i>C difficile</i> toxin
Merz <i>et al</i> (1994) ⁸	0.922 (47/51)	0.988 (639/647)	0.878 (0.038)	Stool sample with request for <i>C difficile</i> toxin
DiPersio <i>et al</i> (1991) ⁷	0.795 (35/44)	0.970 (261/269)	0.773 (0.057)	Stool samples received in laboratory
Schué <i>et al</i> (1994) ⁶	0.961 (49/51)	0.957 (291/304)	0.842 (0.053)	Stool samples received in laboratory

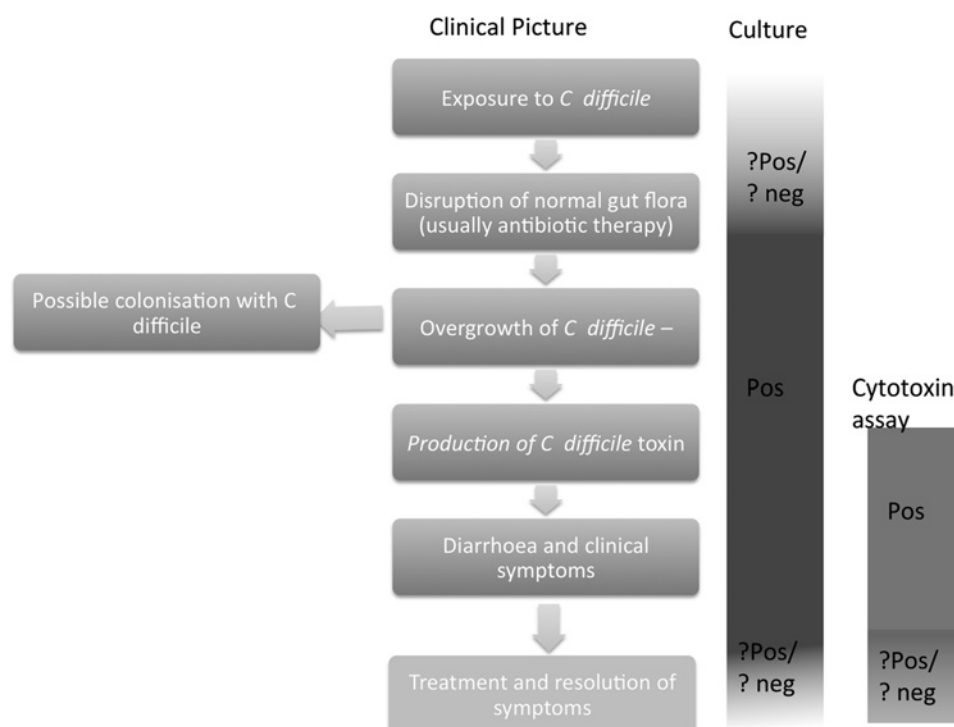
Table 2 Assays for *Clostridium difficile*

Target	Reference method	Other methods
<i>C difficile</i> toxin(s)	Cell-cytotoxicity assay	Toxin enzyme immunoassays; toxin membrane assays
<i>C difficile</i>	Culture	Common antigen (glutamate dehydrogenase) immunoassays
<i>C difficile</i> with the capacity to produce toxin(s)	Cytotoxigenic culture	Toxin gene PCR amplification techniques

Cytotoxigenic *C difficile* was cultured from 2% (4/200) and 1.9% (11/594) of faecal samples from healthy adults in UK¹⁷ and Swedish studies,¹⁸ none of whom had a positive cytotoxin assay. Studies of hospital inpatients typically show higher rates of detection of cytotoxigenic *C difficile*. A US study found 24% (192/810) of symptom-free admissions to have cytotoxigenic *C difficile* cultured on rectal swabs.¹⁹ A UK study found 10% (29/284) of faecal samples from patients admitted to care of the elderly wards were *C difficile* culture positive. A recent hospital admission is a known risk factor for the detection of *C difficile* in faecal cultures.^{15 16}

A number of studies have examined the rate of acquisition of *C difficile* in patients during hospital admission. A US study found *C difficile* in faecal cultures of 10% (65/678) patients admitted to the wards,¹⁶ and this increased to 18% (119/678) by the end of the study. A further US study found asymptomatic carriage of *C difficile* in 7% (29/428) of admissions,²⁰ and this increased to 20% (81/397) of asymptomatic patients, with a further 31 patients developing symptomatic CDI during the study. A study from Boston found an 11% (45/406) rate of culture positivity for cytotoxigenic *C difficile* in new admissions, with a further 15% of initially culture-negative patients acquiring *C difficile* during their hospital stay.¹⁵ Thus, the presence of cytotoxigenic *C difficile* in the faeces of asymptomatic patients is relatively common. The rate of carriage is about 2% in the general population,^{17 18} but rates of 7–25% of hospital admissions are reported depending on the geographic setting of

Figure 1 Pathogenesis of *Clostridium difficile* infection and relationship with reference assays. Pos, positive; neg, negative.



the hospital. It should be noted that most of these studies are more than 10 years old, and that there may have been changes in the epidemiology of the carriage of *C difficile*, notably since the introduction of newer strains and changes in hospital practice.

CLINICAL SIGNIFICANCE OF REFERENCE ASSAYS FOR *C DIFFICILE*

It is clear from the above epidemiological data that the presence of *C difficile* in faeces of asymptomatic patients may create diagnostic difficulties. If in-patients with a high rate of faecal carriage develop diarrhoea, there is a clear possibility that *C difficile* culture will be positive regardless of the cause of the diarrhoea. A number of clinical studies have been designed to investigate the significance of reference assays in the laboratory confirmation of a diagnosis of CDI; these are discussed below.

A 1986 study²¹ looked prospectively at the clinical picture of patients with CDI and compared them with controls. Cases were defined as adults with six unformed bowel motions in 36 h and either stool culture for *C difficile*, positive toxin assay or endoscopic evidence of pseudomembranous colitis. Controls consisted of patients admitted to the same wards with a similar condition, but without diarrhoea. Patients with a positive culture as the only confirmation of CDI were analysed separately from the other cases. Over a year, 149 nine cases (109 positive by CCTA, and 40 that were only *C difficile* culture positive) were compared with 148 controls. The 109 cases confirmed by CCTA had a longer hospital stay before diagnosis, higher rate of underlying disease and hypertension compared with controls. Cases also more commonly had fever and leucocytosis. In contrast, the 40 patients with only a positive culture result were clinically indistinguishable from controls, other than more frequently having fever. Colonoscopy was performed on 96 patients in the study, of whom 39 had evidence of pseudomembranous colitis (PMC). Of the patients with only a positive culture, 3/27 (11%) had endoscopic evidence of PMC. This contrasts with the patients with a positive CCTA of whom 35/68 (51%) had evidence of PMC. From this it could be argued

that only about 20% of diarrhoeal patients that are culture positive but CCTA negative may have CDI. It should be noted that of the 39 patients with PMC on endoscopy, 37/39 had positive culture result and 35/39 had a positive CCTA, a difference that was not significant.

There are only a few studies comparing the results of endoscopy with those of reference assays.^{22–24} Initial studies from the early 1980s found CCTA to be positive in about 90% (27/30) of cases with PMC on endoscopy.^{23 24} In a recent UK study²² flexible sigmoidoscopy was carried out on 179 in-patients with diarrhoea (≥ 3 watery stools in 24 h) of unknown origin. Seventy-nine patients with known CDI were not included in the study. Faecal samples were taken at the time of sigmoidoscopy and the results of CCTA were compared with sigmoidoscopic appearance. PMC was present in 56 patients; of these CCTA was only positive in 27 (48%) of cases. Frozen stool was available from only nine of the 29 CCTA-negative cases with PMC and all of these were TC positive. Though this study appears to show poor sensitivity of CCTA to detect PMC these data are difficult to interpret as patients with known CDI were excluded, patients had diarrhoea for a mean (SD) of 10 (1.2) days before sigmoidoscopy, and at least a third of the patients had received metronidazole before endoscopy. In addition, culture was not universally performed in all patients, and therefore a comparison between reference assays was not made. For these reasons, it is not possible to generalise from these results. While there may be improved sensitivity by performing culture as well as CCTA, further studies are needed to compare the results of endoscopy with laboratory reference assays in CDI. In practice, endoscopy is performed less often than formerly given concerns about its tolerability in typical frail older patients at risk of CDI.

A US study in 1986 looked at the clinical course in untreated patients that were CCTA negative but culture positive.²⁵ Over 11 months, faecal samples were cultured from patients with diarrhoea and CCTA was performed. Physicians were notified of the result of the CCTA, but not of culture. Forty-five patients were included in the study, of whom 16 were culture positive and CCTA positive, and 29 were culture positive and CCTA negative (and outcome data were available for 22 of these). None of the patients that were CCTA negative received specific treatment for CDI. Seventeen of these 22 patients (77%) recovered without treatment. Of the five patients who did not recover, one had a colectomy for inflammatory bowel disease (unrelated to CDI) and the other four died with ongoing diarrhoea, although it is uncertain whether the cause of death was related to CDI. Thirteen patients had further diagnostic studies (barium enema, colonoscopy or sigmoidoscopy) and none of these had any evidence of PMC; six had inflammatory bowel disease and seven had normal examinations.

A UK study in 1995²⁶ examined the clinical picture of patients with faecal samples that were TC positive but negative by CCTA. Forty-one samples of 500 were CCTA positive, and nine samples from seven patients were culture positive but CCTA negative (a further two patients had non-cytotoxigenic *C difficile*). A review of clinical records showed that three of these seven patients had carriage only, with the other four patients having possible disease. Two of those with possible disease had further investigations (colonoscopy or barium enema) that were negative and two further had negative repeat stool samples. The retrospective design of this study makes these estimates likely to overestimate the number of cases of possible disease.

Some other studies have examined the use of reference assays in CDI.^{27 28} However, these studies use laboratory results as part of the definition of CDI, and without clinical follow-up data or

attribution of causality it is not possible to determine the true significance of test results. These studies generally indicate that patients who are culture positive but CCTA negative appear different from those who are CCTA positive.²¹ The great majority of cases do not appear to have CDI^{21 26} and most recover without specific treatment.²⁵ Thus, while there is possibly a small increase in the sensitivity of detection of CDI if TC is used, such gains are likely to be at least offset by the poor specificity of culture-based diagnosis secondary to *C difficile* carriage.

The available evidence is that the two reference assays for the detection of *C difficile* are in fact answering two separate questions. The culture of cytotoxigenic *C difficile* obviously indicates the presence of this organism in faeces, and thus indicates that this particular patient (notably with continuing diarrhoea) may pose a risk of cross-infection to others. However, given the relatively high rates of *C difficile* carriage in hospitalised patients, and the results of detailed clinical studies, a positive culture or TC does not necessarily confirm that the cause of diarrhoea in a particular patient is CDI. In order to answer the question as to whether an individual patient with diarrhoea has CDI then it is necessary to perform a CCTA. It is an oversimplification to ask which of these reference methods is superior as they have different functions. If both reference tests were performed, a positive CCTA would confirm CDI, while negative tests for both would exclude CDI. Faecal samples negative by CCTA but positive by culture may be difficult to categorise, but it appears that the majority of these cases do not have CDI. Thus, clinical assessment of such cases would be important, with careful consideration of alternative diagnoses and reassessment if there is not prompt response to CDI therapy. It may prove impossible for a single reference standard to emerge for the laboratory diagnosis of *C difficile* and a compound definition based on laboratory results and clinical findings will be developed.

ASSESSMENT OF ACCURACY OF DIAGNOSTIC TESTS

There is another important aspect of the use of CCTA and TC as reference assays that indirectly affects the routine laboratory diagnosis of CDI. While CCTA and TC are uncommonly used as frontline methods in diagnostic laboratories, routine tests for CDI are designed, assessed and optimised using these reference assays. It follows, therefore, that as the reference assays are not concordant, then the cut-off threshold used to define positive samples and the reported performance of laboratory tests will vary depending on the reference assay(s) used during development. The accuracy of laboratory diagnostic tests should be measured using reference assays that utilise the same or equivalent targets (see table 2). For example, *C difficile* toxin enzyme immunoassays (EIAs) should be tested with CCTA as a reference standard; if they are measured against TC then accuracy will likely appear to be worse. In general, comparing toxin EIAs with TC will underestimate sensitivity, and comparing with PCR or glutamate dehydrogenase EIAs with CCTA will underestimate specificity. This effect is demonstrated in studies that report comparisons with both reference assays.¹³ There is also no clear rationale for using a different reference assay to attempt to resolve discrepant results in test and reference assays. As the reference assays measure different targets, then use of a second different reference assay on discrepant samples will not improve the assessment of the true accuracy of a diagnostic test. There has been a tendency for laboratories to stop performing the CCTA over recent years, but it is important to maintain the ability to perform this method in some laboratories, not least to be able to validate newer assays.

Take-home messages

- ▶ Different reference methods produce different results for diagnosis of *Clostridium difficile* infection.
- ▶ Cytotoxigenic culture produces more positive results, but this does not necessarily imply a 'better' test.
- ▶ Clarity on the optimum reference (gold) standard method for the laboratory diagnosis of *C difficile* infection requires much larger studies that include relevant clinical data.
- ▶ Selective use of the existing alternative reference (gold) standard methods can provide misleading performance results for new *C difficile* detection methods.

Lack of consistency when investigating the accuracy of diagnostic tests for CDI has contributed to the poor performance of some methods.^{13 29} A further crucial issue here is the use during kit evaluations of samples containing a high proportions of positives relative to that seen in the typical diagnostic setting. This distorts (inflates) the positive predictive values of tests relative to those that are likely to be observed in practice. The positive predictive values of some diagnostic tests are unacceptably low, which will hinder clinical management of CDI and infection prevention and outbreak control, and makes epidemiological data unreliable. Thus, the suboptimal accuracy of routine tests, initially favoured for their convenience in comparison with CCTA and TC, has now led for calls to adopt two-stage methods for the routine laboratory diagnosis of CDI.^{13 29 30}

CONCLUSION

In summary, results obtained from reference assays for the detection of CDI are not identical and answer different questions about *C difficile*. TC identifies patients who are potentially infectious if they have diarrhoea. On available evidence, CCTA is a better test for the laboratory confirmation of CDI, although there needs to be confirmation of whether its sensitivity is improved with additional TC. Patients who are TC positive but CCTA negative, need careful clinical assessment. The majority of these patients do not have CDI^{21 26} and improve without specific treatment.²⁵ When using a reference assay to determine the accuracy of alternative diagnostic tests for CDI, it is important to use a method that targets the same end-point. Further research is urgently needed to improve knowledge of the diagnostic utility and relative performance characteristics of these tests and factors that influence their performance.

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